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The impact of Tunisian Capparidaceae species on cytological, physiological and biochemical mechanisms in lettuce

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ABSTRACT

The Capparidaceae species had excellent phytotoxic effect on weed growth in Tunisia with, regardless to the several perturbation of physiological and cellular processes in target plants. In this study, we report the allelochemicals stress of aqueous (15 g/L) and methanol (6 g/L) extracts of leaves *Capparis spinosa* L. and siliquae of *Cleome arabica* L. on the cytological, physiological and biochemical processes of lettuce. The results showed that aqueous extracts exhibited the mainly cytotoxic effect on root tip cells, with a morphological modification and necrosis phenomena, which correlated with a drastic reduction of mitotic index. In fact, allelochemicals present in these extracts triggered oxidative damage in lettuce manifested by lipid peroxidation as evidenced by increased content of malondialdehyde. In response to this, the disruption in the membrane permeability was revealed by a strong electrolyte leakage. The increase of the antioxidant secondary metabolites was the result of the lyase activity stimulation. In addition, the photosynthetic pigments, including chlorophyll and carotenoids, were maintained by the functional regulation of proline in the cellular levels. These empirical studies consist in valuable bases for more thorough promising studies will help to decipher the allelochemicals; thus, to enhance new natural herbicides mode of action and to help its application on agricultural fields.

1. Introduction

In the course of their evolution, plants have developed mechanisms to cope with and adapt to different types of abiotic and biotic stress imposed by the frequently adverse environment; which are often implicated as very important factors changing allelopathy manifestation in nature (Einhellig, 2004). The plants response to stress depends on the duration, severity and rate of stress imposed (Munne-Bosch and Alegere, 2004). A number of plants have been reported to possess inhibitory effects on the growth and population of neighboring or successional plants by releasing allelochemicals into the soil, either as exudates from living plant tissues or by decomposition of plant residues (Dayan et al., 2000). Action of allelochemicals in target plants is diverse and affects a large number of cytological, physiological and biochemical reactions, which can occur in leaves (Bezemer and van Dam, 2005) and also in roots (Rasmann and Agrawal, 2009). Many studies have shown that membrane perturbations are often suggested the primary site of action of many allelochemicals that trigger further modifications in physiological processes of plant cell. At cellular level, it induces lipid peroxidation, affects some enzymatic activities and rapidly depolarizes the root cell membrane thereby increasing the membrane permeability, thus blocking plant nutrients uptake (Weir et al., 2004). In addition, allelochemicals reduced significantly the defensive abilities of plant by a great distribution in photosynthetic rate, the stratiform structure of the chloroplasts and the nuclei membranes and structure (Peng et al., 2004). These effects reduce plant growth that correlated with a drastic inhibition of the mitotic activity (Sanchez-Moreiras et al., 2008), which is not only significant for the individual plant, but can influence an ecosystem by changing the pattern of vegetation (Rice, 1984).

Cleome arabica L. and *Capparis spinosa* L. belong to a Capparidaceae that are widespread in North Africa, which have high pharmaceutical, economical and ecological values (Musallam et al., 2011). These plants metabolize a large number of secondary metabolites mainly flavonoids, alkaloids, glycosides, organic acids and glucosinolates (Batanouny and Shams, 2006; Ladhari et al., 2013a, 2013b). Some of these compounds have broad spectrum of biological activities and exerts a potent allelopatic effect. In view of the fact that these plants posses a potent phytotoxic effect mainly by the leaves of *C. spinosa* and siliquae of *C. arabica* on weed and crops growth (Ladhari et al., 2013a, 2013b). As no previous studies have been carried out on the mode of action of these plants parts.

This study provides a new understanding of the cytological, physiological and biochemical processes of aqueous and methanol extracts of leaves *C. spinosa* and siliquae of *C. arabica*, in lettuce. The acquisition of

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such knowledge may ultimately provide a rational and scientific basis for the design of safe and effective herbicides.

2. Materials and methods

2.1. Plant materials

Fresh leaves of wild caper (*Capparis spinosa*) and siliquae of spider flower (*Cleome arabica*) were collected identified according to Tunisia flora (Pottier-Alapetite, 1979).

These plants parts were collected during Spring 2010. They were washed several times with tap water and dried in hot-air oven at 60 °C for 72 h. Then, they were cut into 1 cm pieces, powdered in blender and sieved through 40 mesh (420 μm) sieve.

2.2. Preparation of plants extracts

The aqueous and methanol extracts were prepared as described by Ladhari et al. (2013a, 2013b).

2.3. Cytotoxicity test

For germination assays, seeds of *Lactuca sativa* L. were soaked in 5 mL of distilled water on one layer of filter paper in Petri dishes and incubated in germination room [400 μmol photons.m^{-2} s^{-1} photosynthetically active radiation (PAR) at 24/22 °C for 14/10 h light and dark periods]. When the newly emerged roots reached 1.50-2.00 cm in length, they were used in the test. The newly emerged roots were treated with a series of concentrations of aqueous (5, 10, 15 and 20 g/L) and methanol (6 g/L) extracts for 48 h. To avoid toxic effect of solvents, the filter papers were placed in a fume hood for 30 min to allow complete solvent evaporation. Subsequently, 5 mL of distilled water were added to each Petri dish. The control group was treated with distilled water. At the end of each exposure period, root tips were cut and subsequently fixed, macerated, stained and squashed as described previously by Koodkaew et al. (2012) with some modifications. A region approximately 1 cm from the tip was collected and immediately fixed in ethanol/acetic acid (3/1, v/v) for 24 h and transferred to 70% ethanol. Then the roots were macerated for 25 min with 1 N HCl which was used for hydrolysis and maceration. Staining of the chromosome was carried out with acetic carmine for 30 min. One mm of the meristematic zone was immersed in a drop of 45% acetic acid on a clean slide and squashed under a cover-slip by thumb pressure. Three slides were prepared for each treatment and the mitotic figures were evaluated randomly in at least 1000 cells per slide using a light microscope. The mitotic index (MI) was calculated as the proportion of dividing cells (M phase) to the total number of cells observed. The frequency of each mitotic phase was calculated as the percentage in relation to the number of cells in mitosis in the treatment.

2.4. Plant culture and extracts treatment

Seeds of lettuce were germinated in Petri dishes at room temperature in the dark. Seven-day old seedlings were irrigated with distilled water during the first week. Uniform seedlings were subsequently cultured individually in a hydroponic system containing a complete Hoagland's medium (Hoagland and Arnon, 1950) diluted eightfold in a greenhouse (16 h light/8 h dark at 20/17 °C) (Mahmoudi et al., 2011). Individual plants were grown in control (without treatment) and solutions were replaced every third day for two months prior to harvest. After two months of culture, plants are divided into plants cultured in the absence (control) and in the presence of aqueous extracts of leaves *C. spinosa* and siliquae of *C. arabica* prepared at 15 g/L or methanol extracts at the concentration of 6 g/L. After 48 hours of treatment, the plants were harvested and separated into leaves and roots, then weighed before and after drying at 60 °C for 2 days or placed in a freezer (−80 °C) until use. Fresh or dry material is used for the determination of different parameters.

2.5. Chlorophyll and caratenoids content

Hundred mg fresh weight leaves were extracted by 5 mL acetone 80%. Pigments were measured using the spectrophotometric method. Amounts of chlorophyll a (chl a), chlorophyll b (chl b) and total chlorophyll (chl t) and caratenoids (crt) were determined at A_{645}, A_{663} and A_{440} according to Lichtenthaler and Wellburn (1983).

$$crt\left(mg.g^{-1}\right) = (4,7\,A_{440} - (1,38\,A_{663} + 5,48\,A_{645})$$
$$chlt\left(\mu g.mL^{-1}\right) = 20,2*(A_{645}) + 8,02*(A_{663})$$
$$chla\left(\mu g.mL^{-1}\right) = 12,7*(A_{663}) - 2,69*(A_{645})$$
$$chlb\left(\mu g.mL^{-1}\right) = 22,9*(A_{645}) - 4,68*(A_{663})$$

2.6. Electrolyte leakage

Loss of membrane integrity (an indicator of cellular damage) was studied in terms of ion (electrolyte) leakage (EL) which was determined as described by Shalata and Neumann (2001). Fresh leaf and root of lettuce were cut into 2-3 mm pieces and placed in test tubes containing 25 ml of distilled water for 24 h and 48 h in the dark. The initial electrical conductivity of the medium (EC1) was measured. The samples were autoclaved at 120 °C for 20 min to release all electrolytes, cooled down to 25 °C and the final electrical conductivity (EC2) measured. The electrolyte leakage (EL) was calculated from:

$$EL = (EC1/EC2)*100.$$

2.7. Lipid peroxidation

Lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction (Alia et al., 1995). Frozen samples (0.25 g) were homogenized with a mortar and pestle in 2.5 mL of a mixture containing phosphate buffer 67 mM and 0.5 g of PVP (polyvinylpolypyrrolidone). The mixture was centrifuged at 2,000 g for 15 min at 4 °C. An assay mixture containing 2 mL of the supernatant and 2 mL of 0.5% (w/v) TBA in 20% (w/v) TCA was heated at 90 °C for 10 min and then rapidly cooled in an ice bath. After centrifugation (2,000 g for 10 min at 4 °C), the supernatant absorbance was read at 532 nm, and the values corresponding to nonspecific absorption (600 nm) were subtracted. Lipid peroxidation products were measured as the content of TBA-reactive substances. The MDA content (nmol/g FW) was calculated according to the molar extinction coefficient of 155 mM/cm.

2.8. Proline content

The proline (Pro) content was determined using a modified method of Bates et al. (1973). A powder of tissue (10 mg DM) from leaves or roots of lettuce was weighed into 1.5 mL centrifuge tubes, then suspended in 1.5 mL of 3% (w/v) sulphosalicylic acid to precipitate protein. The samples were mixed, centrifuged at 14,000 g for 20 min at 4 °C, and the supernatant transferred to a fresh 1.5 mL tube. An aliquot of 1 mL of supernatant was reacted with 1 mL of glacial acetic acid and 1 mL of ninhydrin reagent (1.25 g ninhydrin in 30 mL of glacial acetic and 20 mL of phosphoric acid 6 M) for 1 h at 100 °C before the reaction was stopped by cooling the tubes in ice. The products were extracted with 2 mL of toluene by vortex mixing, the upper (toluene) phase decanted into a glass cuvette and absorbance read at 520 nm. Pro

concentrations were calculated from the absorbance of a set of Pro standards (0-1 mg/mL) assayed in an identical manner.

2.9. Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia lyase (TAL) activities

Fresh roots or leaves (1 g) were homogenized in a mortar and pestle with 20 mL of cold (4 °C) buffer borate (0.1 M) prepared with pH of 8.7 and 9 for the PAL and TAL activities, respectively. The homogenate was centrifuged at 15,000 tr/min at 5 °C for 10 min. The resulting supernatant was directly used for enzyme assay and mixed with 1 ml of L-phenylalanine (50 mM) (for PAL assay) and L-tyrosine (10 mM) (for TAL assay). The initial optical density (DO1) and the precursor containing the crude enzyme extract were measured, respectively, at 270 nm and 333 nm. After incubation at 40 °C for 90 min, the reactions were stopped by cooling in ice and the optical densities were measured again (DO2).

2.10. Total phenolics content

Total phenolics content was determined using the Folin-Ciocalteau method (Sineiro et al., 1996). Fresh roots or leaves of lettuce (1 g) were extracted with 10 mL of 80% methanol at room temperature. The mixture was centrifuged at 3000 × *g* for 10 min. The supernatant was used to determine total phenolics. One hundred microliter of extract was mixed with 500 μL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 °C for 5 min in the dark. Then, 400 μL of sodium bicarbonate (7.5% (v/v)) solution was added to the mixture. After 90 min at 30 °C, absorbance was measured at 765 nm. Total phenolics content were expressed as mg gallic acid equivalent per g of dry weight using gallic acid calibration curve ($R^2 = 0.971$).

2.11. Total flavonoids content

One gram of samples (fresh roots or leaves) was extracted with 10 mL of 80% aqueous methanol. The mixture was centrifuged for 10 min at 2000 g. Supernatants were used for subsequent analysis. The flavonoid content was measured employing the colorimetric assay described by Quettier et al. (2000), 0.5 mL aliquots of extracts were added with same volume to 2% trichlorure d'aluminium ($AlCl_3$). Absorbance at 430 nm was recorded after 30 min of incubation in the obscurity and flavonoid content was expressed as mg of quercetin equivalent per g of dry weight, using quercetin calibration curve ($R^2 = 0.986$).

2.12. Total alkaloids content

The content of total alkaloids was determined using the Dragendorff reagent (Stumpf, 1984). After homogenate the fresh leaves or roots in methanol (80% (v/v)), a volume of 300 μL of this homogenate was mixed with 100 μL Dragendorff reagent. After centrifugation at 7000 g for 1 min, the pellet was collected and dissolved in 1 mL of NaI (2.45 M). An aliquot of 10 μL of each tube was added to 1 ml of NaI (0.49 M) by following the absorbance is measured at 467 nm. The content of total alkaloids was expressed as mg of papaverine hydrochloride equivalent per g dry weight, using papaverine hydrochloride calibration curve ($R^2 = 0.990$).

2.13. Statistic analysis

The laboratory bioassays and pots culture were conducted in a completely randomized design with three replications. Duncan, Student *t* and ANOVA tests were performed using PASW statistics 18.0, for Windows program, to analyze treatment differences. The means were separated on the basis of least significant differences at the 0.05 probability level.

3. Results

3.1. Mitotic index and frequency of mitotic phases in lettuce roots

The cytotoxicity assays were carried out in the presence of aqueous extracts tested at 5, 10, 15 and 20 g/L, and methanol extracts, at 6 g/L, of *C. spinosa* leaves and siliquae of *C. arabica*, on lettuce seeds germinated for 48 h. Macroscopic examination showed that the aqueous extracts induced a significant inhibition on root growth with morphological changes which were marked by root hairs absence, slight thickening in diameter and burnished end reflecting the necrosis phenomenon. These disturbances were concentration-dependent with strong effect at the highest concentration (Fig. 1A). Although, methanol extracts induced only one morphological change, that is the absence of root hairs (Fig. 1B).

These effects was further confirmed by microscopic studies involving determination of mitotic index reduction, providing definitive information regarding the extent of cytotoxic action. The difference in the values of the mitotic indexes between control and treatments, as well as the percentages of dividing cells in prophase, metaphase, anaphase and telophase, are presented in Table 1. The results showed that the aqueous extracts of *C. spinosa* leaves and siliquae of *C. arabica* had the highest cytotoxic effect on mitotic activity which was concentration-dependent. At the highest concentration, the reduction of dividing cells number was led to significant decrease in the mitotic index of 97.93%, while at the lowest concentration only 36.4% was recorded. Similarly, methanol extracts of *C. spinosa* and *C. arabica* induced an average decline of 70.75% (Table 1).

The decrease in mitotic index was considerably different to mitotic phases which were more or less affected. The telophase was mostly affected by aqueous extracts, showing an average reduction of 42.4% at the highest concentration. While, methanol extracts induced a decrease of 10.77% for prophase compared to the control (Table 1).

3.2. Electrolyte leakage and membrane integrity

The extent of membrane damage in leaves and roots of lettuce was estimated by electrolyte leakage (EL). The aqueous and methanol extracts of *C. spinosa* leaves and siliquae of *C. arabica* influenced significantly the leakage of ions from the roots more than from leaves of lettuce (Fig. 2). Thus for roots, after 24 h, the methanol extracts of *C. spinosa* and *C. arabica* increased significantly the EL to 612% and 740%, respectively. However, an average decrease of these values to 138% and 56% in leaves under the response of *C. spinosa* and *C. arabica* extracts, respectively (Fig. 2). Then, after 48 h, methanol and aqueous extracts of both species influenced slightly the leakage of ions from roots, which was recorded with an average values of 134% and 87.5%, respectively. In leaves, all tested extracts induced an average leakage of 98.1% (Fig. 2).

Increased EL levels indicate that extracts caused stress resulting in disruption of membrane integrity. In order to explore this, the amount of MDA as an indicator of lipid peroxidation was presented in Fig. 3. The methanol extracts of *C. arabica* and *C. spinosa* influenced MDA levels by a respective increase of 127% and 79% in leaves, and 72% and 41% in roots of lettuce. Moreover, aqueous extracts induced an average stimulation of 67% and 39% in roots and leaves of lettuce, respectively (Fig. 3).

3.3. Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities

The activity of PAL and TAL were measured in leaves and roots of lettuce in the presence of aqueous (at 15 g/L) and methanol (6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica* (Fig. 4). These enzymes activities were more or less affected in roots and leaves lettuce by all tested extracts. In roots, PAL activity was inhibited by 30.3% in the

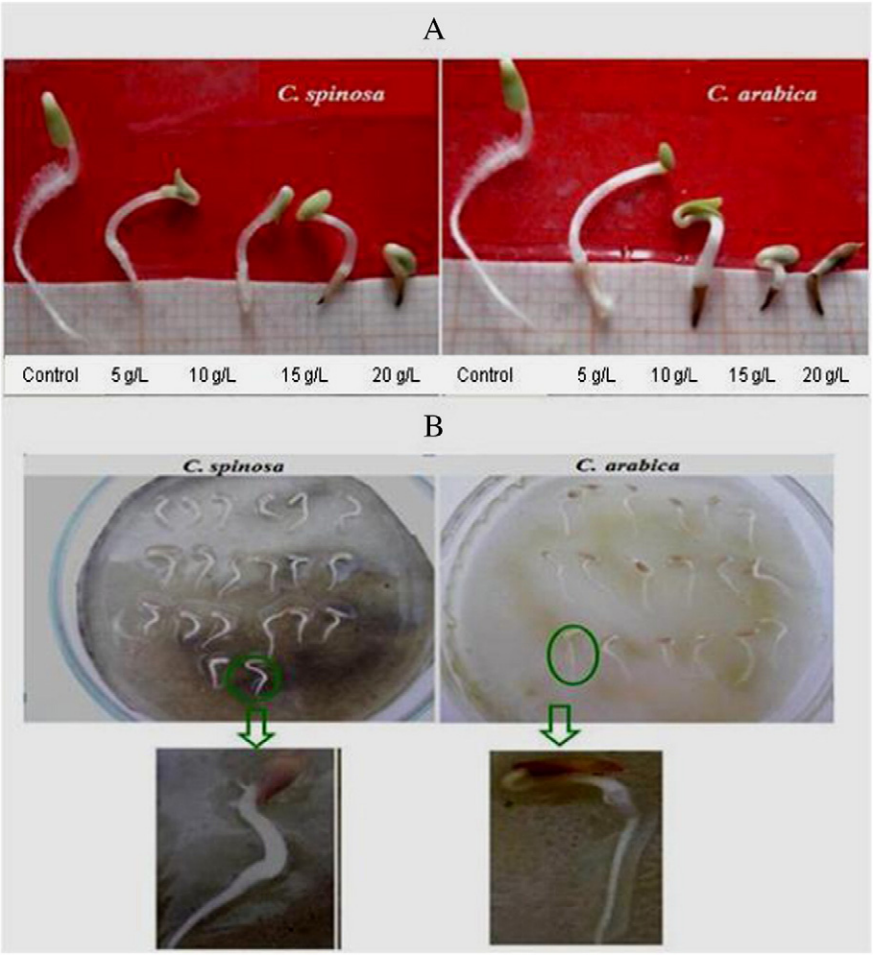

Fig. 1. Effect of (A) aqueous extracts (5, 10, 15 and 20 g/L) and (B) methanol extracts (6 g/L) of leaves of C. spinosa and siliquae of C. arabica, on roots morphology of lettuce.

presence all tested extracts; excepting the methanol extract of *C. spinosa* induced a slight stimulation of 13.8%. In lettuce leaves, the activity of this enzyme was reduced by 27.1% in the presence of methanol extract of *C. spinosa*, and 13.4% in the other cases (Fig. 4A).

In the other side, TAL activity was significantly stimulated by 44% and 81% in roots under treatment with aqueous of *C. spinosa* and *C. arabica*, respectively, and an average stimulation of 59.2% in the presence of methanol extracts. In leaves, TAL activity was near to control in all cases, however a weak stimulation of 32.8% was recorded in the presence of aqueous extract of *C. arabica* (Fig. 4B).

3.4. Proline content

Proline level was measured in roots and leaves of lettuce grown with aqueous and methanol extracts of leaves *C. spinosa* and siliquae of *C. arabica* (Fig. 5). In roots, an average inhibition was recorded of 0.64 times in presence of all tested extracts. However, leaves proline stimulated by 1.42 and 1.76 times in response to the aqueous and methanol extracts application of *C. arabica*. The methanol extract of *C. spinosa* leaves did not influence proline content, while their aqueous extract induced a reduction of 0.78 times (Fig. 5).

Table 1
Mitotic indexes and phases of lettuce meristematic root cells exposed to aqueous (5, 10, 15 and 20 g/L) and methanol extracts (6 g/L) of *C. spinosa* leaves and siliquae of *C. arabica* during 48 h. Means with the same letter in a column are not significantly different at $P < 0.05$ (LSD test). Values (N = 3 ± S.E.).

Extracts		g/L	Cells in mitosis	Mitotic index (mean ± S.E)	Mitotic phase (mean ± S.E)			
					%Prophase	%Metaphase	%Anaphase	%Telophase
Aqueous	*C. spinosa*	Control	481	16.04 ± 1.7a	51.50 ± 2.6a	18.07 ± 1.2c	15.93 ± 3.0a	14.47 ± 2.1a
		5	330	11.01 ± 1.1b	56.85 ± 4.2a	18.82 ± 2.8c	13.48 ± 1.2b	10.83 ± 0.1b
		10	242	8.08 ± 0.7c	51.51 ± 4.9a	19.50 ± 3.2c	14.58 ± 0.9a	14.40 ± 1.9a
		15	209	6.98 ± 1.0d	48.55 ± 3.2b	19.25 ± 3.1c	15.98 ± 1.7a	16.21 ± 0.7a
		20	8	0.26 ± 0.0e	50.00 ± 1.8a	25.00 ± 4.1b	16.66 ± 2.4a	8.33 ± 0.5c
	C. arabica	5	281	9.39 ± 0.3c	53.52 ± 6.1a	22.16 ± 2.1b	12.73 ± 2.5b	11.58 ± 1.1b
		10	270	9.05 ± 0.6c	48.01 ± 2.1b	22.13 ± 1.0b	16.92 ± 1.2a	12.92 ± 0.7b
		15	161	5.39 ± 0.2d	46.25 ± 1.1b	22.64 ± 3.2b	15.32 ± 1.8a	15.77 ± 3.4a
		20	12	0.4 ± 0.0e	48.88 ± 1.6b	28.88 ± 3.5a	13.88 ± 0.7b	8.33 ± 1.1c
Methanol	*C. spinosa*	6	145	4.83 ± 0.6b	48.29 ± 3.2b	32.37 ± 3.9a	10.37 ± 3.4b	8.94 ± 0.9c
	C. arabica	6	136	4.55 ± 0.3b	43.62 ± 2.0b	24.97 ± 2.7b	16.40 ± 2.0a	14.99 ± 2.2a

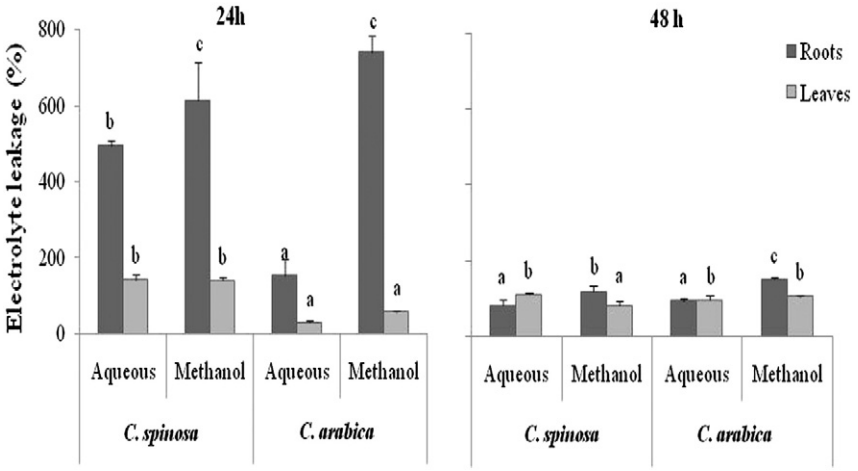

Fig. 2. Effect of aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica*, on electrolyte leakage (%), after 24 h and 48 h of treatments, in lettuce roots and leaves. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

3.5. Chlorophylls and carotenoids content

The aqueous and methanol extracts of *C. arabica* increased the *chlt* by an average stimulation of 1.26 times. This improve is due to the increased content of *chla* by 1.46 times compared to the control. Conversely, aqueous extract of *C. spinosa* reduced *chlt* (17.2%), while the methanol extract had no effect (Fig. 6A).

In the other side, the carotenoids content decreased significantly compared to the control in presence of all cases. Thus, a significant reduction of 0.46 and 0.39 times was recorded under the response of aqueous and methanol extracts of *C. arabica*, respectively (Fig. 6B). Similarly, a respective reduction of 0.6 and 0.9 times was recorded in the presence of *C. spinosa* leaves extracts (Fig. 6B).

3.6. Secondary metabolites production

Secondary metabolites such as total phenols, total flavonoids and total alkaloids possess a considerable role against oxidative stress. The levels of these metabolites were determined in lettuce roots and leaves, which were grown in presence of aqueous (at 15 g/L) and methanol extracts (at 6 g/L) of *C. spinosa* leaves and siliquae of *C. arabica.*

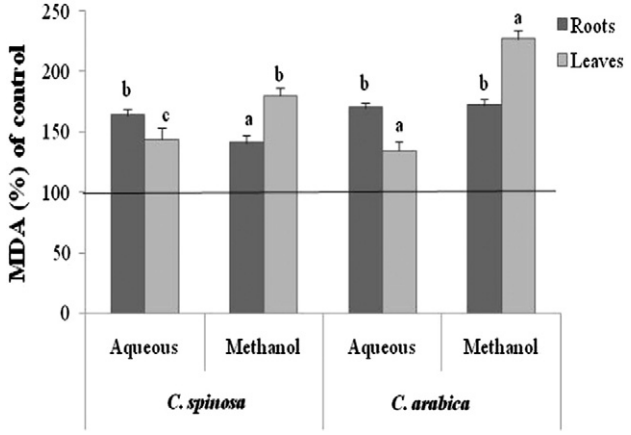

Fig. 3. MDA content expressed in % of control of lettuce (roots and leaves) grown under aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica.* The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

3.6.1. Total phenols content

Total phenols were accumulated significantly in lettuce roots in presence of methanol extracts. Hence, phenols amount was increased by 5.21 and 6.79 times in the presence of methanol extracts of *C. spinosa* leaves and siliquae of *C. arabica*, respectively. These values was recorded respectively of 3.41 and 2.63 under the response of aqueous extracts. In leaves, phenols amount was reduced to 0.64 times in the presence of aqueous extract of *C. arabica* siliquae and in the other cases this value was near to control (Fig. 7).

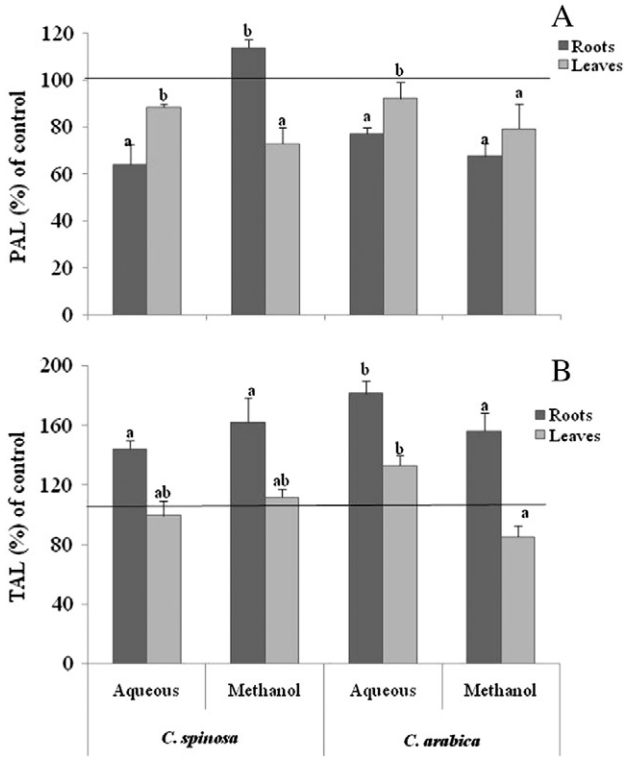

Fig. 4. Effect of aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica*, on (A) Phenylalanine ammonia-lyase (PAL) and (B) tyrosine ammonia-lyase (TAL) activities (% of control) in roots and leaves of lettuce. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

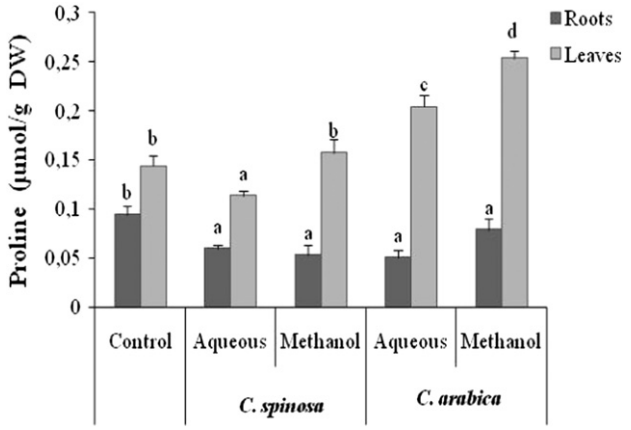

Fig. 5. Effect of aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica*, on proline content (µmol /g^{1} DW) in lettuce roots and leaves. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

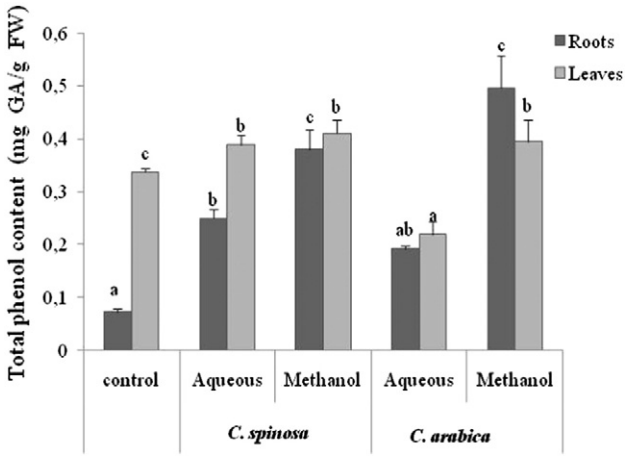

Fig. 7. Total phenols content (mg GA/g FW) of lettuce (roots and leaves) grown under aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica*. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

3.6.2. Total flavonoids content

The accumulation of total flavonoids in roots was significantly improved by 3.38 and 6 times in the presence of methanol extracts of *C. spinosa* leaves and siliquae of *C. arabica*, respectively. This accumulation was respectively increased by 1.35 and 1.88 times in the presence of aqueous extracts. Similarly, in lettuce leaves an average increase of 1.6 times was recorded in the presence of methanol extracts of both plants. However, this accumulation was near to control in the presence of aqueous extracts (Fig. 8).

3.6.3. Total alkaloids content

Alkaloids accumulation in lettuce roots was stimulated by 1.34 times in the presence of *C. spinosa* aqueous extract, however a reduction of 0.62 times was recorded in the other cases. For leaves, this accumulation was reduced by 0.57 times in the presence of aqueous leaves extract of *C. spinosa*, however the aqueous siliquae extract of *C. arabica* induced a stimulation of 1.78 times. In fact, this accumulation was near to control by the methanol extracts (Fig. 9)

4. Discussion

In this study, the aqueous extracts of leaves *C. spinosa* and siliquea of *C. arabica* exhibited the mainly cytotoxic effect on root tip cells, with a morphological modification and necrosis phenomena, which correlated with a drastic reduction of mitotic index. This type of root damage has been reported in the literature as a result of allelochemical stress (Butnariu, 2012). The absence of root hairs was recorded in lettuce treated with leachate of alfalfa (Hedge and Miller, 1992) and with ferulic acid (Caspersen et al., 1999). In addition, Tigre et al. (2012) showed that the organic extracts of *Cladonia verticillaris* induced necrosis in the apex root of lettuce suggesting the accumulation of phenols. Kupidlowska et al. (1994) found that coumarin cause a strong inhibition of roots growth with necrosis phenomena. These morphological changes could be explained by alteration of physiological processes, such as the transport of water or nutrients (Weir et al., 2004) or hormonal disturbance (Demmig-Adams, 1990). According to Bhowmik and Doll (1984), necrosis phenomena may be due to leakage of ions and metabolites owing to altered membrane, causing cell death. This speculated changes in root morphology to a decrease in the mitotic index (Tkalec et al., 2009).

Moreover, these aqueous extracts were considered the major depressor of the mitotic index at the highest concentration. The mitotic index measures the proportion of cells in the M-phase of the cell cycle and its inhibition could be interpreted as cellular death or a delay in the cell proliferation kinetics (Rojas et al., 1993). These results corroborate with those of Sultan and Çelik (2009) who reported that the flower buds aqueous extract of *C. spinosa*, tested at 10, 20 and 30 g/L, induced an inhibitory effect on root growth of *Allium cepa* with significant decrease of mitotic index proportional to the concentration. Ateeq et al. (2002) reported similar observation with commercial herbicides like pentachlorophenol, 2,4-D and butachlor. According to our previous results (Ladhari et al., 2013a, 2013b), the mito-depressive effect could be attributed to the phytotoxic isolated compounds from leaves of *C. spinosa* and siliquea of *C. arabica*. Moreover, this effect was probably

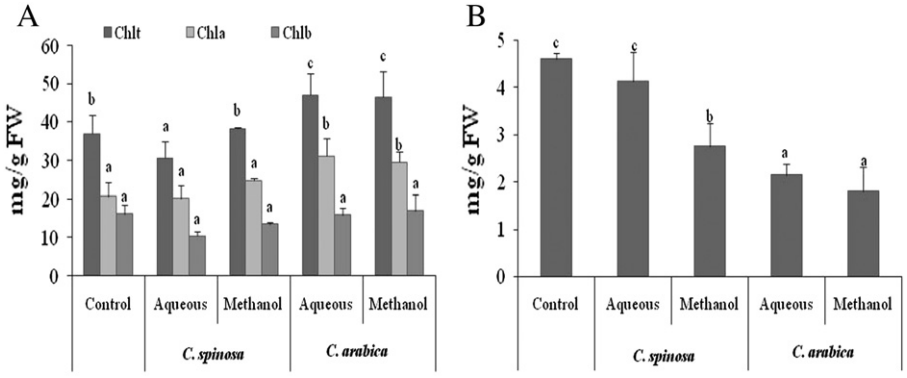

Fig. 6. Effect of aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica*, on (A) chlorophyll and (B) carotenoid content (mg/g FW) of lettuce. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters in columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

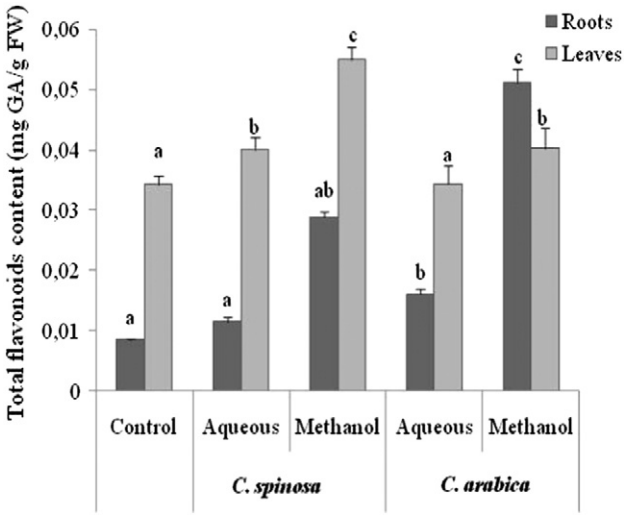

Fig. 8. Total flavonoids content (mg QE/g FW) of lettuce (roots and leaves) grown under aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica*. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

due to inhibition of DNA synthesis or a blocking in the G2 phase of the cell cycle, preventing the cell form entering mitosis (Sudhakar et al., 2001).

This cytotoxic effect could be due to the increased levels of electrolyte leakage resulting in disruption of membrane integrity. A decrease in membrane permeability could be due to peroxidation of polyunsaturated fatty acids in the biomembranes resulting in the formation of several by products, including malondialdehyde (MDA) (Maness et al., 1999). Recently, it has been demonstrated that electrolyte leakage measurements may be correlated with several physiological and biochemical parameters conditioning the plant responses to environmental conditions (Vainola and Repo, 2000). In fact, cell membranes are one of the first targets of many allelochemicals stresses, which were reported in the literature. Wang et al. (2009) showed that leaves leachate of *Jatropha curcas* induced an allelopathic stress by increasing the electrolytes leakage in roots of marigolds. More recent studies show that the increase in electrolyte leakage from *Echinochloa crus-galli* roots under *Cymbopogon citratus* essential oil stress is the result of a disruption of membrane integrity (Poonpaiboonpipat et al., 2013). This increase is more obvious in roots lettuce denoting that this part was susceptible to the phytotoxic effect of allelochemicals. This result corroborate with Reigosa et al. (2001) who reported that the hydroxamic acid (BOA) stress increased significantly the electrolytes leakage from lettuce roots after 4 h more than 8 h.

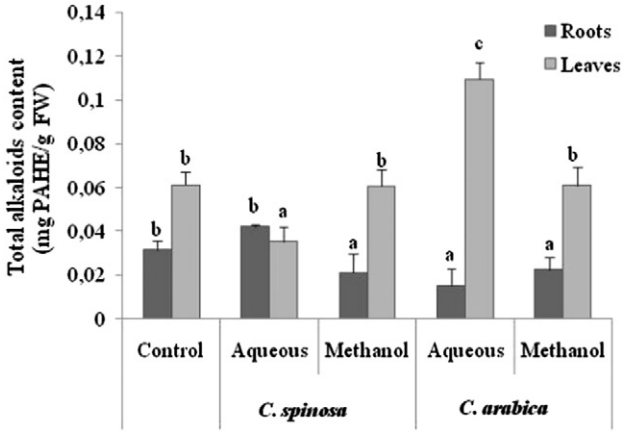

Fig. 9. Total alkaloids content (mg PAHE g/FW) of lettuce (roots and leaves) grown under aqueous (at 15 g/L) and methanol (at 6 g/L) extracts of *C. spinosa* leaves and siliquae of *C. arabica*. The bars on each column show standard error. Values (N = 5 ± S.E.). Different letters on columns indicate significant differences among treatments at $P < 0.05$ (LSD test).

In the current study MDA levels in roots and leaves of lettuce were significantly enhanced upon treatment with aqueous and methanol extracts of leaves *C. spinosa* and siliquae of *C. arabica*. Studies on cucumber and sorghum roots showed that injury to membranes under the influence of allelopathic compounds were proportional to lipid peroxidation measured as MDA content (Zeng et al., 2008). The stimulation of MDA level by *Chrysanthemum morifolium* aqueous extract in *Chrysanthemum morifolium* leaves was recorded by Zhou et al. (2009), which disturbed the balance between the activity of anti-oxidative enzymes and peroxidation of membrane lipids and accordingly affected the structure and functions of membranes, the main mechanisms of allelopathy (Singh et al., 1999). According to Gmerek and Politycka (2011), ferulic and *p*-coumaric acids evoke the lipids peroxidation in the roots of maize, pea and radish. The process of lipid peroxidation consists of three stages: initiation, propagation and termination (Catala, 2006). The initiation phase of lipid peroxidation is the abstraction of hydrogen atoms from lipid molecules. Several free radicals are responsible for this, one being hydroxyl radical (Gutteride, 1988). Peroxidation of lipids is particularly damaging because the products of this process lead to the spread of further free radical reactions (Catala, 2009). Dependence between enzymatic activity and lipid peroxidation was also observed in soybeans subjected to allelopathic stress by treatment with phenolic extract from *Brassica napuse* (Haddadchi and Gerivani, 2009).

The enzymatic activities (PAL and TAL) was responded differently to allelochemical stress, either by inhibition or stimulation in the target organs. These enzymes catalyzes reactions pathways in phenylpropanoid metabolism, which are known by their protective role. Raifa-Hassanein et al. (2005) also showed that TAL activity was distinct from that of PAL in the callus of *Nicotiana tabacum* and *Hibiscus sabdarifa*, respectively. According to Wajahatullah et al. (2002), this activation depends on the species, genotype, environmental conditions and the availability of endogenous substrates. Few studies have been carried out on the effects of allelochemicals on PAL activities, and results are contradictory. For example, Sato et al. (1982) pointed out that ferulic acid was ineffective on the PAL of sweet potato (*Ipomea batatas*) and pea (*Pisum sativus*). In contrast, with these authors, Dos Santos et al. (2004) demonstrated that PAL activities increased in roots of soybean under treatment with ferulic acid. Simultaneous activation of PAL is considered as product-specific, such as for lignin or tannin or anthocyanin biosynthesis (Singh et al., 2009). However, maintaining TAL activity after the dramatic fall in that of PAL was a real relay in the synthesis of these compounds (Dogbo et al., 2012).This could be explained by the fact that TAL activity was estimated by the formation of *p*-coumaric acid. Indeed, the *p*-coumaric acid may result from two biosynthetic pathways: the direct deamination of tyrosine and the transformation of *E*-cinnamic acid produced by the PAL, as suggested by Rösler et al. (1997). Consequently, *p*-coumaric acid remains a crossroad in the synthesis of most phenylpropanoids (Berner et al., 2006).

The accumulation of the proline level was observed mainly in lettuce leaves in response to the methanol siliquae extract of *C. arabica*. However, in roots this accumulation was reduced in the presence of all tested extracts. Generally, metabolic studies indicate that most of the proline accumulated in plants in response to stress is the result of enhanced synthesis from glutamate (Hare and Cress, 1997). Proline may also be synthesized from ornithine (Delauney et al., 1993). A decrease in proline oxidation frequently accompanies the onset of stress (Madan et al., 1995) although this in itself is unlikely to account for the levels of proline often accumulated (Chiang and Dandekar, 1995). According to Singh and Rao (2003), proline protects proteins from denaturation by maintaining the hydration level. The increase of proline content was recorded in

Tagetes erecta under treatment with leachate of *Jatropha curcas* (Wang et al., 2009). Similar result was recorded by Abdulghader and Nabat (2008) who reported that with application of heliotrope leaves extracts, level of proline significantly increased in leaves of Dodder. More recently, Ibrahim et al. (2013) showed that the aqueous leaves extract of corn increase the accumulation of proline in wheat leaves. In addition, this accumulation under allelochemicals stress was also reported in the literatures. The significant accumulation of proline increased significantly in *Triticum turgidum* and in *Pisium sativum* in response to a respective application of coumarin (Abenavoli et al., 2006) and phenolic compound (Batish et al., 2007).

Chlorophylls and carotenoids are crucial plant pigments for photosynthesis and their abundance results in greater assimilation of solar radiations into consumable sugars. In our study, the reduction chlorophyll a, chlorophyll b and total chlorophyll content in lettuce treated with aqueous leaves extract of *C. spinosa* agrees with earlier reports. Ilori et al. (2007) reported a reduction in these photosynthetic pigments in *Amaranthus cruentus* L. and *Oryza sativa* L. seedlings treated with aqueous extract of *Tithonia diversifolia*. Similarly, Ahmed et al. (2004) reported that the root and shoot extracts of *Chenopodium murale* reduced chlorophyll and protein contents of *Melilotus indicus*, *Trifolium alexandrium*, *Triticum pyramidal*, *Lycopersicon esculentus* and *Cucumus sativa*. Leaf photosynthetic capacity depends on physiological characteristics such as chlorophyll contents, rubisco activity and photo system efficiency (Bowes, 1991). These reports have clearly shown that allelochemicals in plant extracts are capable of impairing chlorophyll synthesis thereby reducing chlorophyll accumulation. According to Yang et al. (2004) as well as Singh et al. (2009), allelochemicals in plant extracts may reduce chlorophyll accumulation in three ways viz inhibition of chlorophyll biosynthesis, stimulation of chlorophyll degradation or both. Therefore, the allelochemicals present in the tested extracts species exerted growth inhibitory effects in the lettuce growth through reduction in chlorophyll synthesis. The results obtained are in agreement with those of Jaleel et al. (2008) and Al-Sobhi et al. (2006).

Carotenoids are pivotal accessory pigments playing major roles in photosynthesis (Demmig-Adams, 1990) by collecting light and transferring the excitation energy to the chlorophyll (Siefermenn-Harms, 1987) and by stabilizing proteins of the light-harvesting complex (Plumley and Schmidt, 1987). In addition, these pigments are responsible for quenching of singlet oxygen (Knox and Dodge, 1985). In our results, the decrease of carotenoids is in agreement with earlier study which reported that responses of receiver plants is due to allelochemicals present in plant extracts. Ibrahim et al. (2013) reported that the carotenoid content was significantly decreased in wheat in presence of aqueous extract of *Zea mays*. Root exudates from sorghum were reported to inhibit the activity of hydroxyphenyl pyruvate dioxygenase which resulted in plastoquinone deficiency and therefore, disrupt the biosynthesis of carotenoids (Meazza et al., 2002). Romagni et al. (2000) reported that usnic acid is a strong inhibitor of phytoene desaturase which converts phytoene to carotenoids.

The secondary metabolites production is influenced by biotic and abiotic stresses (Ferreira, 2007). These metabolites provide a protective role (Zeng et al., 2008). In this study the enhanced accumulation of total phenols, flavonoids and alkaloids in roots and leaves of lettuce were recorded as a response to aqueous or methanolic extracts. These results are corroborate with Djanaguiraman et al. (2005) who reported that allelochemicals present in leaves leachate of *Eucalyptus* increased the phenol content in sorghum and bean. Thus, the disturbance of the physiological processes was correlated positively with the enhanced accumulation of some allelochemicals (Omezzine et al., 2012). Also, Garrido et al. (2012) reported that the inhibitory effect of olive aqueous extract on sunflower root growth was positively correlated with the accumulation of total flavonoids, and Lola-Luz et al. (2013) noted that the accumulation of flavonoids in cabbage reflects the inhibitory effect of the commercial extract of brown seaweed (*Ascophyllum nodosum*).

5. Conclusion

Based on this study, it is concluded that lettuce parts responded differently to the treatment with aqueous and methanolic leaves extracts of *C. spinosa* and siliquae of *C. arabica*. These extracts evoked cytotoxic, biochemical and physiological disturbances, which causing a main inhibition on root growth. The cytotoxic effect caused morphological changes and necrosis phenomena, which correlated with a drastic reduction of mitotic index (MI). In leaves, a slight disturbance was recorded in the pigment levels, but carotenoid was more affected, in addition of the disruption in membrane permeability, revealed by a strong electrolyte leakage with an increase in lipid peroxidation. Furthermore, lettuce parts have accumulated secondary metabolites with antioxidant properties such as polyphenols and flavonoids. This accumulation was the result of the lyase activity stimulation, especially the TAL, involved in their biosynthesis. Thus, more studies are needed to enhance new natural herbicides mode of action and to assist its application on agricultural fields.

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